Total Phenolics Concentration and Antioxidant Potential of Extracts of Medicinal Plants of Pakistan

Ammar Saleem [a,*], Markku Ahotupa [b] and Kalevi Pihlaja [a]

[a] Department of Chemistry, University of Turku, FIN-20014 Turku, Finland.
Fax: +358 2 3336700. E-mail: amsale@utu.fi

[b] MCA Research Laboratory, Department of Physiology, University of Turku, Tykistökatu 6, FIN-20520 Turku, Finland

* Author for correspondence and reprint requests

Z. Naturforsch. **56 c,** 973–978 (2001); received July 19/August 23, 2001

Phenolics, Pakistani Medicinal Plants, Antioxidants

Thirty-seven plant organs, traditionally used as drugs, collected in Pakistan, were extracted with 70% acetone and analyzed for their total phenolics concentration and antioxidant potential. Seven extracts showed more than 85% inhibition of lipid peroxidation *in vitro* as compared with blank. Butylated hydroxytoluene (BHT) (IC_{50} = 233.6 μg / l ± 28.3) was the strongest antioxidant in our test system. The IC_{50} results indicate that the extracts of *Nymphaea lotus* L. flowers, *Acacia nilotica* (Linn.) Delile beans, *Terminalia belerica* Roxb. fruits, and *Terminalia chebula* Retz. (fruits, brown) were stronger antioxidants than α-tocopherol, while *Terminalia chebula* Retz. (fruit coat), *Terminalia chebula* Retz. (fruits, black) and *Ricinus communis* L. leaves were weaker antioxidant extracts than α-tocopherol and BHT. Total phenolics concentration, expressed as gallic acid equivalents, showed close correlation with the antioxidant activity. High performance liquid chromatographic analysis with diode array detection at 280 nm, of the seven extracts indicated the presence of hydroxybenzoic acid derivatives, hydroxycinnamic acid derivatives, flavonol aglycones and their glycosides as main phenolics compounds. This information, based on quick screening methods, enables us to proceed towards more detailed chemical and pharmacological understanding of these plant materials.

Introduction

Phenolic compounds of natural origin are of recent interest for improving human health and in this context, crude extracts of a variety of plant materials are being investigated for their phenolics and antioxidant potential (Haslam and Cai, 1994; Larson, 1988). The antioxidant activity of phenolics is mainly due to their redox potential that is determined with a variety of assays (Diaz *et al.*, 1995). Phenolics also contribute significantly to the color, taste and flavor of many foods and drinks and their nutritive value is of interest as possible alternatives of synthetic antioxidants (Cynshi *et al.*, 1995).

The World Health Organization estimates that the population of developing countries relies to about 80% on traditional medicines for their primary health needs and 85% of these traditional medicines involve the use of plant extracts. This means that about four billion people rely exclusively on plants as their source of medication (Fransworth, 1988).

A wide variety of folk medicinal plants, growing on fertile land of Pakistan, are unknowingly exploited and have not been scientifically investigated for their biologically active principles (Kirtikar and Basu, 1975). Although some of these plant materials have been in focus for the drug discovery and a wealth of information is available in pharmacology literature about their therapeutic applications but a few of them have been collected from Pakistan and studied for their possible pharmacological applications. In this context, as a part of systematic study of Pakistani folk medicinal plants, we selected some known and some not so well known plant organs from their natural habitats in Pakistan, identified them and determined their phenolic contents and antioxidant potential. This study, based on quick recognition of phenolics of selected plant materials and a correlation between their phenolic contents and antioxidant potential in their crude extracts, is a first step forward to reveal the phytochemistry of these plant materials.

0939–5075/2001/1100–0973 $ 06.00

Experimental

Plant collection, identification and documentation

Thirty-seven plant organs, belonging to 22 plant families, were collected from their natural habitats. Plant organs were identified by Professor Zahoor Ahmed, Department of Botany, Punjab University, Lahore, Pakistan. The collected plants organs were air dried under shade and packed in sealed plastic bags and transported to and stored in the cold storage of the Department of Chemistry, University of Turku, for further studies. The plant specimens were deposited in Turku University Herbarium TUR, (Table I).

Chemicals

Folin reagent, Na_2CO_3, and EDTA were purchased from Fluka BioChemica, Buchs, Switzerland). Butylated hydroxytoluene, α-tocopherol and t-butyl hydroperoxide (t-BuOOH) (70% aqueous solution); caffeic acid, chlorogenic acid, gallic acid, catechin, rutin, and naringenin were from Sigma Chemical Co. (St. Louis, MO) and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was from Bio-Orbit Ltd. (Turku, Finland). All other chemicals for extraction and HPLC analysis were of HPLC grade except acetone which was of analytical grade.

Table I. Pakistani medicinal plants included in the study.

Entry	Nomenclature[a]	Family	Voucher number (TUR)	Part extracted
1	*Cassia fistula* Linn.	Caesalpinioideae	354097	leaves
2	*Cassia occidentalis* Linn.	Caesalpinioideae	354099	leaves
3	*Lawsonia inermis* L.	Lythraceae	354106	leaves
4	*Nymphaea lotus* L.	Nymphaeaceae	354109	flowers
5	*Solanum nigrum* L.	Solanaceae	354112	leaves
6	*Bauhinia variegata* L.	Ceasalpinioideae	354095	leaves
7	*Mentha viridis* L.	Labiatae	354107	leaves
8	*Morus alba* L.	Moraceae	354108	leaves
9	*Corchorus antichorus* Raeusch.	Tiliacae	354102	Beans
10	*Carum carvi* L.	Umbelliferae	354096	fruits
11	*Cassia fistula* Linn.	Caesalpinioideae	354118	flowers
12	*Citrullus colocynthis* (L.) Schard.	Cucurbitaceae	354101	seets
13	*Foeniculum vulgare* Mill.	Umbelliferae	354104	seets
14	*Volutarella divarciata* Bth.	Compositae	354115	flowers
15	*Coriandrum sativum* L.	Umbelliferae	354119	seets
16	*Cordia myxa* L.	Bonvolvulaceae	354103	seets
17	*Peganum harmala* L.	Zygophyllaceae	354110	seets
18	*Acacia nilotica* (Linn.) Delile.	Mimosoideae	354094	beans
19	*Terminalia belerica* Roxb.	Combretaceae	354122	fruits
20	*Terminalia chebula* Retz.	Combretaceae	354114	fruits (brown)
21	*Terminalia chebula* Retz.	Combretaceae	354114	fruit coat (brown)
22	*Terminalia chebula* Retz.	Combretaceae	354113	fruits (black)
23	*Lawsonia inermis* L.	Lythraceae	354120	fruits
24	*Cassia fistula* L.	Caesalpinioideae	354089	beans
25	*Cichorium intybus* L.	Compositae	354100	roots
26	*Euphorbia helioscopia* L.	Euphorbiaceae	354125	roots
27	*Glycyrrhiza glabra* L.	Fabaceae	354105	roots
28	*Ricinus communis* L.	Euphorbiaceae	354121	leaves
29	*Sapindus detergens* Roxb.	Sapindaceae	354111	fruits
30	*Acacia nilotica* (Linn.) Delile.	Mimosoideae	354123	leaves
31	*Azadirachta indica* (L.) A. Juss.	Meliaceae	354124	leaves
32	*Ricinus communis* L.	Euphorbiaceae	354126	leaves
33	*Solanum nigrum* L.	Solanaceae	354127	fruits
34	*Polygonum bistorta* L	Polygonaceae	354116	roots
35	*Curcuma longa* L.	Zingiberaceae	354117	rhizomes
36	*Ziziphus jujuba* (Lam.) Mill.	Rhammnaceae	354128	fruits
37	*Onosma echioides* Clarke	Boraginaceae	354129	leaves

[a] Nasir E. and Ali S. I. (1972), Flora of West Pakistan, Fakhri Printing Press, Karachi, Pakistan.

Extraction

Each dried plant material (500 mg) was homogenized for 40 s with Ultra-Turrax T25 (Janke and Kunkel, IKA-Labortechnik, Germany) in 20 ml of 70% acetone at room temperature. The homogenate was continuously stirred for 1 hr followed by centrifugation for 10 min at 4000 × *g*. The pellet was extracted twice with 25 ml of 70% acetone and the extract was evaporated to dryness under reduced pressure at 35 °C by a rotary evaporator (Laborota 4000, Heidolph, Germany). Each dried extract was diluted in deionized water at 40 mg ml^{-1} and the extracts were stored at −18 °C for further analyses.

Total phenolics concentration (TPC)

The assay was slightly modified for this study by homogenizing the contents at 1500 × *g* for 10 min and measuring the absorbance at 730 nm instead of usual 765 nm. Each extract (0.1 ml) was mixed with 5.9 ml water and 1.0 ml of the diluted extract was mixed with 1.0 ml Folin reagent. The mixture was allowed to stand for 2–5 min and 2 ml of 20% Na_2CO_3 was added. The mixture was stirred and incubated for 10 min at room temperature, homogenized at 1500 × *g* for 8 min and its absorbance was measured at 730 nm on Perkin-Elmer Spectrophotometer 550. Standard curve was drawn with known concentrations of gallic acid. The results were calculated according to the mathematical procedure of Segal (1975).

Antioxidant Activity (AOA)

Rat liver microsomes were prepared as follows: Livers from adult male Sprague-Dawley rats were excised and chilled in ice-cold 0.25 M sucrose solution. A 20% (weight/volume) liver homogenate was prepared in sucrose at 0 °C with Potter-Elvehjem glass-Teflon homogenizer at 500 r.p.m. The homogenate was centrifuged at 12000×*g* for 10 min (at 4 °C). The pellet was discarded and the supernatant fraction was further centrifuged at 105 000×*g* for 60 min (at 4 °C). The resulting microsomal pellet was resuspended in 0.15 M KCl.

Luminometric measurements were carried out on Bio-Orbit Luminometer (Bio-Orbit, Turku, Finland) equipped with a specially programmed personal computer for the assay. Six samples were simultaneously analyzed in reaction cuvettes by automatic dispension of *t*-BuOOH. A 800 µl of buffer (50 mM Na_2CO_3 + 0.1 mM EDTA, pH = 10.2), 6 µl luminol (0.5 mg / ml), 20 µl of rat liver microsomes in 0.15 M KCl (final concentration, 1.5 µg protein / ml), and 20 µl of the samples were pipetted into reaction cuvettes. The reaction was initiated by 0.9 mM *t*-BuOOH at 33 °C. Deionized water was used as blank. IC_{50} (concentration that inhibits lipid peroxidation by 50%) was calculated on the basis of integral (area under the kinetic reaction curve) during 30 min runs.

HPLC analysis

HPLC measurements were carried out mainly according to our earlier studies (Saleem *et al.*, 2001). Each extract was filtered through Millex-HV_{13} filter, 0.45 µm (Millipore, Bedford, MA) before injecting into the HPLC system. Separations were performed on LiChroCart® (250 × 4.6 mm i.d., 5 µm, Merck, Darmstadt, Germany). HPLC system consisted of Pump L-7100 connected with Diode Array Detector L-7455 and a programmable Autosampler L-7250, (Hitachi Ltd., Tokyo, Japan). Two solvents were used for elution: (A), acetonitrile; (B), 5% formic acid. The elution profile was 0–5 min., 100% B (isocratic); 5–60 min., 0–30% A in B (linear gradient). The flow rate was maintained at 1.0 ml min^{-1} with column back pressure of 70–136 bar. The wavelength of diode array detector was fixed at 280 nm.

Results and Discussion

The aim of the present study was the assessment of phenolic contents and the determination of the antioxidant activity of the extracts of folk medicinal plant organs from Pakistan with the help of simple and efficient assays.

The Folin-Ciocalteau method was chosen due to its sensitivity, lower interference and quickness to quantify the phenolics as compared to competitive tests (Amerine and Ough, 1974; Waterman and Mole, 1994). This test quantifies the phenolics by reaction of Folin-Ciocalteau reagent with all molecules containing hydroxyl groups (Torres *et al.*, 1975). For the determination of total phenolics, the Folin-Ciocalteau assay was slightly modified. Homogenization of the contents immediately before measuring the absorption noticeably reduced

the turbidity and 730 nm proved to be a more suitable absorbance wavelength and gave higher total phenolic concentration as compared to 765 nm. These minor changes provided better reproducibility in triplicate measurements in our test conditions, (Saleem *et al.*, 2001).

The antioxidant activity test was carried out to assess the ability of the extracts to inhibit lipid peroxidation *in vitro*. The assay was mainly based on the study by Ahotupa *et al.* (1997). Fourteen extracts exhibited moderate inhibition (31.5% to 77%) and seven extracts showed strong inhibition (84% to 94%) antioxidant activity as compared with blank (deionized water, inhibition 0.03% at maximum). The seven extracts, showing stronger antioxidative activity, were further tested to find out their IC_{50} values to be comparable with BHT and α-tocopherol. For the determination of IC_{50}s, the data analysis was carried out by nonlinear least square curve fit at four-parametric competition curves. Results (based on IC_{50} data) showed that the extracts of *Nymphaea lotus* L. flowers, *Acacia nilotica* (Linn.) Delile beans, *Terminalia belerica* Roxb. fruits, and *Terminalia chebula* Retz. (fruits, brown) are stronger antioxidants in our test system than α-tocopherol, while *Terminalia chebula* Retz. (fruit coat), *Terminalia chebula* Retz. (fruits, black) and *Ricinus communis* L. leaves are weaker antioxidant extracts than BHT and α-tocopherol. The IC_{50} values showed the following order; BHT, 233.6 μg / l ± 28.3 > *Nymphaea lotus* L. flowers, 397.3 μg / l ± 7.3 > *Acacia nilotica* (Linn.) Delile beans, 422.3 μg / l ± 29.8 > *Terminalia belerica* Roxb. fruits, 553.3 μg / l ± 38.9 > *Terminalia chebula* Retz. (fruits, brown), 529.5 μg / l ± 25.0 > α-tocopherol, 834.1 μg / l ± 26.3 > *Terminalia chebula* Retz. (fruit coat, brown), 1016.7 μg / l ± 29.4 > *Terminalia chebula* Retz. (fruits, black), 1954.1 μg / l ± 57.2 > *Ricinus communis* L. leaves, 2494.0 μg / l ± 330.2 (Fig. 1).

Total phenolics concentration results, compared with those of antioxidant activity, indicate that extracts with high antioxidant activity have higher concentration of phenolics (Fig. 2). However, no direct linear dependence between the phenolics concentration and the inhibition percentage was obtained mainly due to the non-specificity of Folin-Ciocalteau test. This indicates that a more detailed chemical analysis of the selected extracts will reveal strong antioxidant phenolic compounds or synergistic effects between certain constituents.

This study also reports major groups of phenolic compounds found in seven extracts showing

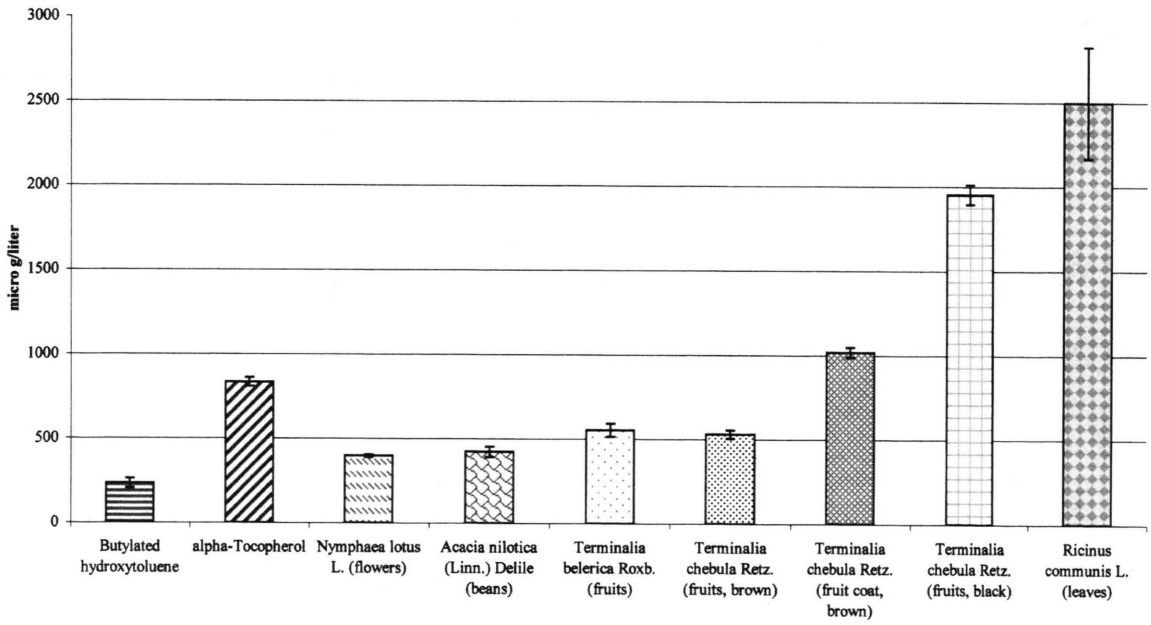

Fig. 1. Antioxidant potential of Pakistani medicinal plant extracts, butylated hydroxylotuene, and alpha-tocopherol. Results (IC_{50}) are average values SD, $N = 3$.

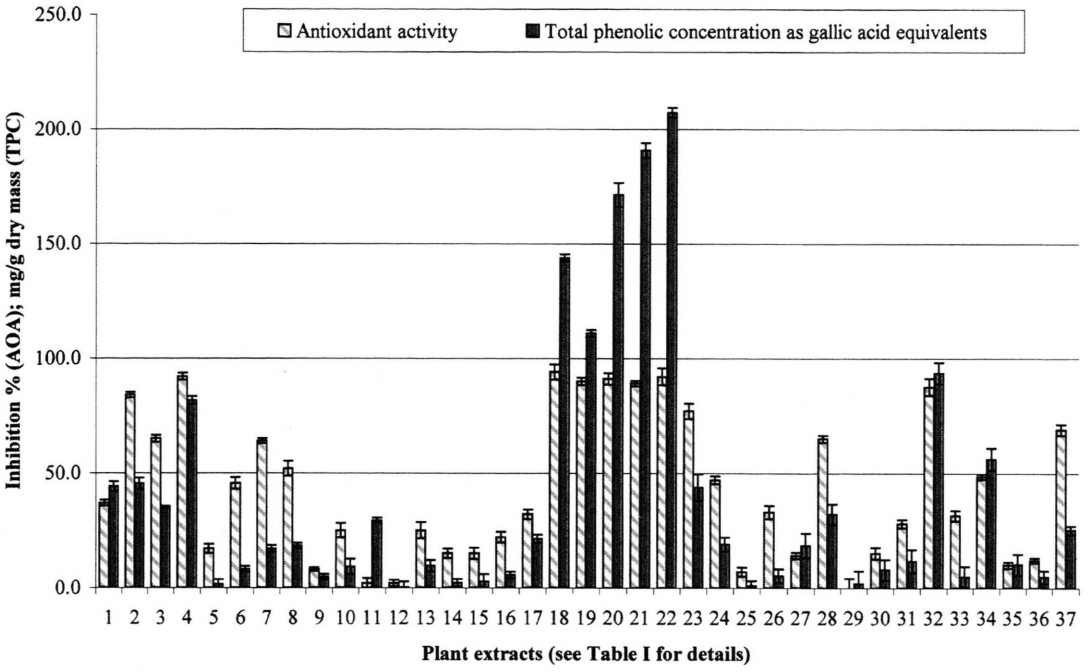

Fig. 2. Antioxidant potential (AOA) and total phenolics concentration (TPC) of Pakistani medicinal plants extracts. Results are mean values SD, $N = 3$.

Table II. Identification of major phenolics of Pakistani medicinal plant extracts by HPLC-DAD. [a]

Extract analyzed	Peak number	Retention time [min]	UV max, [nm]	Major phenolics [b, c]
Nymphaea lotus L. (flower)	10	42.05	280	flavonol (aglycone)
	14	46.29	280	flavonol (aglycone)
Acacia nilotica (Linn.) Delile (beans)	2	5.70	285	HBA
	3	6.48	284	HBA
	48	38.82	285	HBA
	13	46.33	281	flavonol (aglycone)
Terminalia belerica Roxb. (fruits)	1	6.32	315	HCA
	6	48.82	314	HCA
	7	49.34	255,300sh,365	flavonol glycoside
	8	50.07	253,303sh,360	flavonol glycoside
Terminalia chebula Retz. (fruits, brown)	34	26.39	285	HBA
	52	49.11	305	HCA
Terminalia chebula Retz. (fruit coat, brown)	8	11.68	282	HBA
	11	21.42	275	flavonol (aglycone)
	14	27.10	257sh,290,325sh	flavonone
Terminalia chebula Retz.(fruits, black)	6	31.42	250,302sh,328	flavonol glycoside
	24	45.80	265,305sh,355	flavonol glycoside
	26	48.76	264,305sh,356	flavonol glycoside
Ricinus communis L. (leaves)	11	13.64	282	HBA
	27	49.58	256, 301sh, 366	flavonol glycoside
	29	52.13	262, 300 sh, 359	flavonol glycoside

[a] HPLC conditions are mentioned in the *Experimental* section; [b] HBA = Hydroxybenzoic acid derivative; [c] HCA = Hydroxycinnamic acid derivative.

higher total phenolic concentration and antioxidant activity with the help of HPLC-DAD. Phenolic compounds were identified on the basis of retention times, the shapes and UV maxima, (Table II). The UV spectra of the selected peaks, compared with those of standards, indicated the presence of hydroxycinnamic, hydroxybenzoic acid derivatives, and flavonol glycosides. Hydroxycinnamates were identified on the basis of UV spectra of caffeic acid and chlorogenic acid, while hydroxybenzoic acids were compared with gallic acid. Flavonol aglycones were recognized by comparing their UV spectra with that of catechin, and flavonol glycosides with rutin while flavanone in *T. chebula* (fruits, coat brown) was recognized by comparison with the naringenin spectrum.

In conclusion, this study has formed the basis for selecting the most active antioxidant extracts with higher phenolic contents, subjecting them to detailed chromatographic and spectroscopic analysis for revealing their molecules responsible for the potent antioxidant activity.

Acknowledgements

This study was funded by Finnish Cultural Foundation and Juvanita Pharma OY. The authors would like to thank Dr. Zahoor Ahmed, former Professor of Taxonomy, University of the Punjab, Lahore, Pakistan for identifying the plant materials under study.

Ahotupa M., Mäntylä E. and Kangas L. (1997), Antioxidant properties of the triphenylethylene antiestrogen drug tormifene. Naunyn-Schmiedeberg's Arch. Pharmacol. **824**, 1–6.

Amerine M. A. and Ough C. S. (1974), Wine and Must Analysis. Wiley, New York, pp. 115–117.

Cross C. E., Halliwell B., Borish E. T., Pryor W. A., Amae B. N., Saul R. L., McCord J. M. and Harman D. (1987), Oxygen radicals and human disease. Annu. Intern. Med. **107**, 526–545.

Cynshi O., Takashima Y., Katoh Y. and Tamura K. (1995), Action of phenolic antioxidants on various active oxygen species. J. Biolumin. Chemilumin. **10**, 261–269.

Diaz N., Sánchez F. G. and García J. A. (1995), Enhancement and inhibition of luminol chemiluminescence by phenolic acids, J. Biolumin. Chemilumin. **10**, 175–184.

Fransworth N. R. In: Wilson E. O (Ed.) (1988), Biodiversity. National Academy Press, Washington D. C., pp. 83–97.

Haslam E. and Cai Y. (1994), Plant polyphenols (vegetable tannins): gallic acid metabolism. Nat. Prod. Rep. **11**, 41.

Kirtikar K. R. and Basu B. D. (Eds.) (1975), Indian Medicinal Plants, Vol. I., pp. 157–189.

Larson R. A., (1988), The antioxidants of higher plants. Phytochemistry **27**, 969–978.

Saleem A., Jyrki L., Kalevi P. and Elina O. (2001), Effects of long-term open-field ozone exposure on leaf phenolics of European silver birch (*Betula pendula* Roth). J. Chem. Ecol. **27**, 1049–1062.

Segal I. H. (1975), Biochemical Calculations. Wiley, New York, pp. 134–145.

Torres A. M., Mau-Lastovicka T. and Rezaaiyan R. (1987), Total phenolics and High Performance Liquid Chromatography of phenolic acids of avocado. J. Agric. Food. Chem. **35**, 921–925.

Waterman P. G. and Mole S. (Eds.) (1994), Analysis of Phenolic Plant Metabolites, Blackwell Scientific, Oxford, pp. 116–133.